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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 39/00

A2

(11) International Publication Number: **WO 00/06194**

(43) International Publication Date: 10 February 2000 (10.02.00)

(21) International Application Number: PCT/US99/17190

(22) International Filing Date: 29 July 1999 (29.07.99)

(30) Priority Data:
09/124,744 29 July 1998 (29.07.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications
US 08/795,925 (CIP)
Filed on 5 February 1997 (05.02.97)
US 09/137,956 (CIP)
Filed on 5 February 1998 (05.02.98)
US 09/124,744 (CIP)
Filed on 29 July 1998 (29.07.98)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: DEPLETION OF CELLS RESPONSIBLE FOR ANTIBODY-MEDIATED GRAFT REJECTION

(57) Abstract

The present invention provides compositions, and methods of using said compositions, which are useful to reduce allogeneic or xenogeneic graft rejection. The invention provides methods and compositions for promoting in an animal of a first species a state of tolerance against Galα1,3Gal epitopes present on a xenograft from an animal of a second species, thereby preventing hyperacute rejection (HAR) of the xenograft. The methods and compositions according to the invention cause the elimination or anergy of specific lymphoid cells which are responsible for the production of xenoreactive natural antibodies (XNAs) which cause HAR of the xenograft. The invention also provides immunotoxin compositions and methods of using the same which are useful to reduce allogeneic or xenogeneic antibody-mediated graft rejection. The methods and compositions according to the invention cause the reduction or elimination of specific cells responsible for the production of antibodies that cause graft rejection.

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DEPLETION OF CELLS RESPONSIBLE FOR ANTIBODY-MEDIATED GRAFT REJECTION

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the depletion or down-modulation of cells responsible for allogeneic and xenogeneic antibody-mediated graft rejection.

Summary of the Related Art

Organ transplantation has become a well-established clinical procedure. However, there are still at least two major problems that need to be resolved in order to provide a satisfactory outcome to all potential transplant recipients. These are (a) the lack of available donor organs, and (b) the need for long-term immunosuppression to prevent graft rejection.

The need for organs has continued to rise during the past years. Currently, there are more than 33,000 Americans waiting for organ transplants, but only about 4,800 organs donated each year. Because of this growing gap, xenogeneic organ transplantation is an increasingly important area of interest.

Size, physiological similarities, availability and ethical issues have made the pig one of the best studied organ donor species for xenotransplantation (Sachs, D. H. 1992, "MHC-Homozygous Miniature Swine" in Swine as Models in Biomedical Research, Swindle, M.M. et al. (Eds.) (Iowa State University Press, Ames, Iowa, 1992), p. 3; Cooper, D.K.C. et al., 1991, "The Pig as Potential Organ Donor for Man" in Xenotransplantation, Cooper, D.K.C. et al., (Eds.) (Springer-Verlag, Heidelberg, Germany, (1991) p. 481).

Graft rejection may be a consequence of either, or both, cell-mediated and antibody-mediated events. Based upon results of histopathology tests, graft rejection has been characterized to be hyperacute, acute or chronic. Antibody-mediated rejection may be involved in all of these stages of rejection.

(Ch. 13 in Cellular and Molecular Immunology, 3rd Edition, Abbas, A.K. et al., (Eds.) (Sounders Co., Philadelphia, PA)).

Hyperacute rejection (HAR) is characterized by rapid thrombotic occlusion of the graft vasculature that begins within minutes to hours after host blood vessels are anastomosed to graft vessels. HAR is mediated by antibodies which pre-exist in naive hosts, the so-called "natural antibodies", which bind to endothelium and activate complement. Antibody and complement induce a number of changes in the graft endothelium that promote intravascular thrombosis. The endothelial cells are stimulated to secrete high molecular weight forms of von Willebrand factor that mediate platelet adhesion and aggregation. Both endothelial cells and platelets undergo membrane vesiculation, leading to shedding of lipid particles that promote coagulation. Endothelial cells lose their surface heparin sulfate proteoglycans that normally interact with anti-thrombin III to inhibit coagulation. Complement activation also leads to endothelial cell injury and exposure of sub-endothelial basement membrane proteins that activate platelets. These processes contribute to thrombosis and vascular occlusion, and the organ suffers irreversible ischemic damage within a matter of hours. (Cellular and Molecular Immunology, *supra*).

Acute vascular or delayed graft rejection, like hyperacute rejection, is characterized by interstitial edema and hemorrhage; however, in acute vascular rejection the extent of thrombosis is more pronounced, and there is an infiltrate consisting of mononuclear leukocytes and neutrophils. Acute vascular rejection is observed in both allografts and xenografts. While the mechanisms underlying acute vascular rejection are not well understood, it is considered that natural antibodies play a significant role. It is believed that elimination of natural antibodies will facilitate the engraftment of transplanted bone marrow cells thus enhancing the ability for the formation of a state of mixed bone marrow chimerism which will lead to the induction of specific immune tolerance to the donor organ transplant, whether it be an allograft or a xenograft. Such induction of specific immune tolerance to a donor organ transplant could allow the

transplant recipient to avoid the use of immunosuppressive agents and other long-term debilitating treatments and in the case of xenotransplantation overcome the deficiency in the number of donor organs available for organ transplants. (Cellular and Molecular Immunology, *supra*).

Natural antibodies are believed to arise as a consequence of exposure to cross-reactive microbial antigens. In the allogeneic setting, the natural antibodies are directed towards the red blood cell surface antigens, described as the ABO antigens. Differences in the ABO system between donors and recipients limit blood transfusions and organ transplants by causing antibody and complement-mediated dependent cell lysis of cells expressing the blood group antigens. Today, hyperacute rejection by anti-ABO is not a clinical problem because all graft donors and recipients are selected to have the same ABO type. However, hyperacute rejection of allografts may still occur as a consequence of the presence in the graft recipient of natural antibodies directed against other alloantigens. In human ABO-incompatible kidney transplantation, preoperative depletion of natural antibodies has allowed successful long-term function of allotransplants with similar survival rates as ABO-compatible allografts. It has been shown that depletion of natural antibodies for a non-defined period may lead to a state in which the graft survives even if the antibodies return to the recipient circulation. This phenomenon is described as "accommodation" (Platt et al., (1991) Immunology Today 11:250-456) and might reflect a change in the affinity or specificity of the returning antibodies, a change in the expression of endothelial cell antigens in the donor organ or a change in the susceptibility of the endothelial cells to injury. (Cellular and Molecular Immunology, *supra*).

Xenogeneic natural antibody-mediated hyperacute rejection is a very significant barrier to xenotransplantation (Platt J.L. and Bach, F.H. (1991) Transplantation 52:937). Overcoming this barrier is important to the long-term success of pig-to-primate xenotransplantation. Recent studies have demonstrated that a predominant epitope on porcine cells recognized by human natural antibodies is a carbohydrate that includes a terminal galactose residue in the

conformation of the galactosyl α-1,3 galactose disaccharide structure (Neethling, F.A. et al. (1994) Transplantation 57: 959; Ye, Y. et al., (1994) Transplantation 58: 330; Sandrin, M. S. et al. (1993) Proc. Natl. Acad. Sci. USA 90: 11391; Good et al. (1992) Transpl. Proc. 24: 559). Immunopathologic analysis of tissue samples from organs undergoing hyperacute rejection reveals the presence of recipient natural antibodies and complement components along the endothelial surfaces of blood vessels (Leventhal, J.R. et al. (1993) Transplantation 55: 857; Leventhal, J.R. et al. (1993) Transplantation 56:1; Platt, J.L. et al. (1991) Transplantation 52: 214; Platt, J.L. et al. (1991) Transplantation 52: 1037). Galili et al., J. Exp. Med. 160:1519-1531 (1984), teaches that these anti-gal antibodies represent 1-4% of the total IgM and 1% of the total IgG in primates. Sandrin et al., Proc. Natl. Acad. Sci. USA. 90:11391-11395 (1993), teaches that it is the IgM XNAs, most of which are specific for Galα1,3Gal, which initiate the activation of human complement on porcine cells. The Galα1,3Gal epitope is synthesized by the addition of a terminal galactosyl residue to a pre-existing galactose residue linked to N-acetyl-glucosaminyl residue. The reaction is catalyzed by the glucosyltransferase UDP galactose: β-D-galactosyl-1,4-N-acetyl-D -glucosaminide α-1,3 galactosyltransferase (α1,3GT). In species expressing α1,3GT, natural antibodies reactive against the galactosyl α1,3 galactose moiety are absent. The lack of α1,3GT in humans, apes and Old World primates results in a failure to express the galactosyl α1,3 galactose epitope, making the presence of natural antibodies reactive to this epitope permissible. It has been shown in mice, a species that normally expresses the galactosyl α1,3 galactose epitope, that disruption of murine α1,3GT gene by embryonic stem cell technology leads to the development of natural antibodies reactive against the galactosyl α1,3 galactose epitope (Thall A. et al., (1995) J. Biol. Chem. 270: 21437; Thall A. et al., (1996) Transplant. Proc. 28: 561).

Three major approaches have been explored in pig-to-primate experimental models in an effort to overcome hyperacute rejection (HAR). In one approach, recipient natural antibodies are depleted by organ perfusion or by

immunoadsorption over Galα1,3Gal immunoaffinity columns and hyperacute rejection of the transplanted organ is delayed or does not occur. Dintzis and Dintzis, Proc. Natl. Acad. Sci. USA (1992) 89:1113-1117, teaches that ongoing T cell independent and T cell dependent immune responses can be immunosuppressed when an appropriate dose of antigen is administered. Coutts et al., (Lupus (1996) 5(2):158) and Hachmann et al., (Bioconjug. Chem. (1994) 5(5):390) teach a composition for treating the autoimmune disease systemic lupus erythematosus comprising a chemically defined conjugate having a non-immunogenic backbone that is effective for inducing tolerance to autoantigens involved in lupus. Kiessling et al. (WO 96/20236) has described a polyglycomer with unique biological properties relative to monomeric ligands, but does not suggest a tolerizing effect. Sablinski et al., (1997) Surgery 121: 381 teaches that the challenge is to develop effective compositions and methods for inducing such tolerance to the Galαl,3Gal epitope. However, the natural antibodies return to the pre-removal levels very rapidly (less than one week) with resulting rejection of the organ. In the other two approaches, the depletion or inhibition of complement, or the use of organs from genetically engineered pigs transgenic for human complement regulatory proteins have also been successfully used to overcome HAR. However, in almost every case, all of these manipulations have been combined with heavy pharmacologic immunosuppressive therapy which, although ineffective alone, extends graft survival if HAR is prevented by one or more of the above three approaches. Nevertheless, in those graft recipients that have survived the therapeutic regimen, graft failure has resulted almost invariably from what is believed to be an antibody-mediated form of rejection rather than cellular rejection. Latinue et al., (1993) Transplant Proc. 250:336.

A preferred approach, which would provide a solution to all forms of rejection, is the induction of both T and B cell tolerance. Induction of specific immune T and B cell tolerance may be achieved through the use of mixed bone marrow chimerism (Sachs, D. H. (1995) "Mixed Chimerism as an Approach to Transplantation Tolerance" in Transplantation Immunology, Bach, F.H. and

Auchincloss, H. Jr. (Eds.) Wiley-Liss, New York, p. 219) or alternatively, through the use of genetically engineered autologous bone marrow transplants (US Patent No. 5,614,187). Both of these protocols in xenotransplantation include the step of removing the Galα1,3Gal antibodies prior to the transplantation of the bone marrow. It is considered that homing of the bone marrow cells to the recipient bone marrow and subsequent engraftment would be facilitated if the natural antibodies were prevented from returning during the induction phase.

Depletion of cells may be achieved by a variety of procedures but usually involves a cell specific binding reagent linked to a functional moiety, e.g. antibodies or immunotoxins. An immunotoxin is generated through the linkage of an antibody specific for a target cell antigen with a cytotoxic substance such as the toxin ricin. Upon parenteral injection, its antibody portion, usually the antigen binding region, directs the immunotoxin to the target and its toxic portion destroys the target cell. Immunotoxins have been developed primarily in the unrelated field of cancer research as targeted therapeutic agents combining a ligand – e.g., an antibody or a growth factor that binds with partial or complete selectivity to a target cell –, coupled to either bacterial or plant toxins to target neoplastic cells in a specific fashion. Hence, Ghetie, M.A. and Vitetta, E.S. (1994) Current Opinion in Immunology 6: 707, teaches that immunotoxins directed against the common B-chronic lymphoblastic leukemia -(CLL) antigen, B-cell pre-lymphocytic leukemia (PLL), non-Hodgkin's lymphoma (NHL) antigens, CD6 and human small cell lung cancer (SCLC) antigens have been engineered and found to be effective to various degrees against their respective target cells. Flavell et al. (1995) Hematological Oncology 13: 185 teaches the use of anti-CD7 and anti-CD38 bispecific immunotoxins for the delivery of the toxin saporin, a ribosome inactivating protein, to a human T cell acute lymphoblastic leukemia (T-ALL) cell line thereby inhibiting its proliferation.

Recent studies have been directed to the optimization of the use of immunotoxins almost exclusively for cancer therapy purposes. Flavell et al. (1995) Int. J. Cancer 62: 337, teaches that therapy of human B cell lymphoma-bearing

SCID mice is more effective with anti-CD19-saporin and anti-CD38-saporin immunotoxins used in combination than with either immunotoxin used alone. O'Connor et al. (1995) Blood 86:4286, on the other hand, teaches that anti-CD19-ricin immunotoxin synergizes with doxorubicin (a cytotoxic anthracycline antibiotic isolated from cultures of *Streptomyces peucetius varcaesius*, and also marketed as ADRIAMYCIN™ (Adria Laboratories, Dublin OH) and etoposide (VEPESID™, Bristol-Myers Squibb) on multidrug-resistant and drug-sensitive tumors. Similarly, Ghetie et al. (1996) Int. J. Cancer: 68: 93, teaches the use of two immunotoxin constructs containing either anti-CD22 or anti-CD19 antibodies and deglycosylated ricin A chain (dgA) in combination with any one of three chemotherapeutic drugs: doxorubicin, cyclophosphamide (CYTOXAN™, Bristol-Myers Squibb) or camptothecin (an anti-tumor alkaloid) for the treatment of SCID mice with advanced B cell neoplasia. Furthermore, Ghetie et al. (1992) Blood 80: 2315, teaches that the anti-tumor activity of an anti-CD22 immunotoxin in SCID mice with disseminated Daudi lymphoma is enhanced by either an anti-CD19 antibody or an anti-CD19 immunotoxin.

Thomas et al. (1997) Transplantation 64:124 reports that pre-clinical studies of allograft tolerance in rhesus monkeys have shown that the administration of an anti-CD3-immunotoxin peri-transplant with donor bone marrow induces operational tolerance to kidney allografts. Anti-CD3 immunotoxins do not, however, affect ongoing antibody responses, such as natural antibodies. Therefore, this anti-CD3 immunotoxin would not be effective in a pre-sensitized graft recipient expressing natural antibodies.

Despite the impressive progress made in the field of immunotoxin research as a cancer therapy, there has been no corresponding progress in the use of immunotoxins in the field of organ transplantation. For example, there has been no suggestion as to how to eliminate those cells that produce natural antibodies in connection with transplantation rejection. There is, therefore, a need to develop effective compositions and methods for eliminating those antibody producing cells which result in either hyperacute or acute vascular rejection while allowing

the donor cells to engraft and induce tolerance to the donor organ graft antigens.

BRIEF SUMMARY OF THE INVENTION

The present invention provides compositions, and methods of using said compositions, which are useful for reducing allogeneic or xenogeneic graft rejection. The invention provides methods and compositions for promoting in an animal of a first species a state of tolerance against Galα1,3Gal epitopes present on a xenograft from an animal of a second species, thereby preventing hyperacute rejection (HAR) of the xenograft. The invention provides down-modulatory and preferably immunotoxic compositions, and methods of using the same, which are useful for reducing the levels of natural antibodies during the induction phase of tolerance induction. The methods and compositions according to the invention cause the reduction or elimination (down-modulation) of specific cells responsible for the production of antibodies, the presence of which results in hyperacute or acute vascular rejection.

In a first aspect, the invention provides immunotoxins comprising a cell-specific antigen binding moiety, together with a backbone or carrier moiety, and a down-modulator in an operable linkage. Cell-specific antigen-binding moiety immunotoxins according to this aspect of the invention comprise the structure:

$$(AB)_n - Y$$

wherein AB represents a cell-specific antigen binding moiety plus a backbone or carrier, Y represents a down-modulatory functional moiety, n is a number from 1 to 2, and – represents an operable linkage, provided, however, that when AB is directed against the CD19, CD38, CD22 or CD7 antigen, then Y is not saporin, that when the AB is directed against CD19, Y is not deglycosylated ricin A chain, and that when the AB is directed against CD22, then Y is not *Pseudomonas* exotoxin A.

In one preferred embodiment, AB is directed against specific cells that will differentiate into cells producing antibodies that cause graft rejection. Preferably, the AB is directed against the CD5 or cell surface IgM and/or IgG antigens.

In another preferred embodiment, AB is directed towards cells that produce antibodies that cause graft rejection. In certain preferred embodiments, AB is directed against B cell specific target antigens. In certain other preferred embodiments, AB is directed against plasma cell specific target antigens. Most preferably, AB is directed against the CD38, CD22, or HM1.24 antigens, PC-1 family members (PC-1: phosphodiesterase I; EC 3.1.4.1/nucleotide pyrophosphatase; EC 3.6.1.9), or Syndecan-1(CD138) family members, provided that when AB is directed against CD38, then Y is not saporin, and that when AB is directed against CD22, then Y is not saporin or *Pseudomonas* exotoxin A.

In yet another embodiment of the invention, AB consists of bispecific antibodies with each of the two AB arms recognizing different antigens.

In certain preferred embodiments of this aspect of the invention, AB comprises a backbone or carrier having a molecular weight from about 5,000 Da to about 1,000,000 Da. In certain other preferred embodiments, Y is an agent having a molecular weight from about 5,000 Da to about 1,000,000 Da.

In preferred embodiments according to this aspect of the invention, Y is an agent that down-modulates, and preferably is cytotoxic to, the function of specialized cell types involved in processes responsible for graft rejection. Preferably, Y is an agent that down- modulates, and more preferably is cytotoxic to, the function of plasma cells or B cells. Preferred agents, Y, according to this aspect of the invention include, without limitation, ceramides, lipophilic toxins, radioisotopes, diphtheria toxin, plant toxins, ribosome inactivating proteins, anti-IgM, anti-IgG and the antineoplastic antibiotic agent doxorubicin (ADRIAMYCIN™ (Adria Laboratories, Dublin OH)). In certain preferred embodiments, Y is a ribosome inactivating protein such as saporin, or pokeweed antiviral protein. In certain particularly preferred embodiments, Y is a lipophilic

toxin selected from N,N-dimethylsphingosine (DMS), N,N,N,-trimethylsphingosine (TMS), and ceramide analogs. In yet other preferred embodiments of the invention, Y is a radioisotope, preferably selected from the group consisting of ^{131}I, ^{90}Yt, and Tc.

The operable linkage in the down-modulatory composition according to the invention includes any association between AB and Y that allows AB to bind its antigen and Y to exert a down-modulatory effect on the cell type to which AB is bound. One preferred operable linkage is a covalent linkage between AB and Y. In certain preferred embodiments, the covalent linkage is directly between AB and Y, so as to integrate Y into the backbone. Alternatively, the covalent linkage may be through an extended structure such as an oligosaccharide, glycolipid, autologous IgG, or an aliphatic hydrocarbon chain. Another preferred operable linkage is a lipophilic association between AB and Y. In certain preferred embodiments, AB is contained in a liposome and Y is covalently linked to a lipophilic molecule and thereby is associated with the liposome.

In certain other preferred embodiments, AB and Y are bound to, or associated with, a carrier molecule. Preferably, the carrier molecule is a neoglycoprotein, a glycoprotein, a peptide, a glycolipid, autologous IgG, or a synthetic carrier. In certain preferred embodiments, the neoglycoprotein is bovine serum albumin or human serum albumin. In certain other preferred embodiments, the glycolipid is selected from 1,2-di-O-hexadecyl-sn-glycero-3-phospho-ethanolamine (HDPE), ceramide, and ceramide analogs. In yet other preferred embodiments of the invention, the synthetic carrier is Galα1,3Gal–HDPE or Galα1,3Gal–polyacrylamide.

In certain preferred embodiments, the down-modulatory immunotoxin composition further comprises an inhibitor of protein kinase C (PKC) or an inhibitor of a member of the Bcl-2 family. Preferably, the PKC or Bcl-2 family member inhibitor is a sphingosine. More preferably, the sphingosine is N,N-dimethylsphingosine or N,N,N-trimethylsphingosine (TMS). In certain other preferred embodiments, the down-modulatory immunotoxin composition further

comprises a small molecule inhibitor of B cell activation. Preferably, the small molecule inhibitor inhibits *syk-*, *lyn-*, *Btk-*, *blk-*, *fyn-*, and/or *shc*-mediated activation of signal transduction.

In a second aspect, the invention provides a method for facilitating in a transplant recipient of a xenogeneic or allogeneic organ a reduction or down-modulation of those cells responsible for allograft or xenograft antibody mediated graft rejection. The method according to this aspect of the invention comprises administering to the transplant recipient a down-modulatory amount of a down-modulatory immunotoxin composition comprising the structure

$$(AB)_n - Y$$

wherein AB represents a cell-specific antigen binding moiety plus a backbone or carrier, Y represents a down-modulatory functional moiety, n is a number from 1 to 2, and – represents an operable linkage.

In certain preferred embodiments according to this aspect of the invention, the method further comprises administering an inhibitor of protein kinase C (PKC) or an inhibitor of a member of the Bcl-2 family. The PKC inhibitor or inhibitor of a Bcl-2 family member may be administered simultaneously with the $(AB)_n$–Y immunotoxin or may be administered separately. Preferably, the PKC or Bcl-2 family member inhibitor is a sphingosine. More preferably, the sphingosine is N,N,-dimethylsphingosine or N,N,N-trimethylsphingosine (TMS).

In certain other preferred embodiments according to this aspect of the invention, the method further comprises administering, either simultaneously or separately, a small molecule inhibitor of B cell activation. Preferably, the inhibitor of B cell activation inhibits *syk-*, *lyn-*, *Btk-*, *blk-*, *fyn-*, and/or *shc*-mediated activation of signal transduction.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the specificity of the anti-Gal ELISPOT assay using baboon spleen cells.

Figure 2 shows the specificity of the anti-Gal ELISPOT assay using GalT(-/-) mouse spleen cells.

Figure 3 demonstrates the effect on treatment of GalT(-/-) mice with anti-IgM $F(ab')_2$ on the frequency of anti-Gal IgG and IgM secreting spleen cells.

Figure 4 exhibits the effect on treatment of GalT(-/-) mice with anti-IgM $F(ab')_2$ on the frequency of total IgG and IgM secreting spleen cells.

Figure 5 depicts that anti-CD38-RCA abrogates anti-Gal production *in vitro*.

Figure 6 depicts the Galα1,3Gal antibody (XNA) profile of the baboon B75-23 that was subjected to immunoaffinity adsorption.

Figure 7 shows the circulating anti-CD38 levels in the baboon administered anti-CD38-dRCA immunotoxin.

Figure 8 shows the baboon anti-mouse response in the baboon administered anti-CD38-dRCA immunotoxin.

Figure 9 shows the circulating Galα1,3Gal antibody (XNA) in the baboon administered anti-CD38-dRCA immunotoxin demonstrating that the anti-CD38-dRCA immunotoxin abrogates XNA production.

Figure 10 establishes that DMS containing liposomes completely inhibit *in vitro* anti-Galα1,3Gal antibody production.

Figure 11 establishes that DMS-containing liposomes completely inhibit *in vivo* anti-Galα1,3Gal antibody production in GalT(-/-) mice.

Figure 12 shows the effect of treatment of GalT(-/-) mice and baboon B36-46 spleen lymphocytes with anti-IgM $F(ab')_2$ on the frequency of anti-Gal IgG and IgM secreting spleen cells.

Figure 13 shows that a combination of anti-IgM and dimethylsphingosine eliminates the anti-Gal antibody production by baboon cells *in vitro*.

Figure 14 demonstrates that anti-CD22 (RFB4) cross-reacts with both human and baboon spleen B cells.

Figure 15 shows that a representative anti-CD22 immunotoxin according to

the invention mediates inhibition of antibody production by baboon cells.

Figure 16 shows that baboon antibody secreting cells are depleted by incubation with anti-CD22 dg-Ricin A immunotoxin.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides compositions, and methods of using said compositions, which are useful for reducing allogeneic or xenogeneic graft rejection. The invention provides methods and compositions for promoting in an animal of a first species a state of tolerance against Galα1,3Gal epitopes present on a xenograft from an animal of a second species, thereby preventing hyperacute rejection (HAR) of the xenograft. The invention provides down-modulatory and preferably immunotoxic compositions, and methods of using the same, which are useful for reducing the levels of natural antibodies during the induction phase of tolerance induction. The methods and compositions according to the invention cause the reduction or elimination (down-modulation) of specific cells responsible for the production of antibodies, the presence of which results in hyperacute or acute vascular rejection.

The compositions and methods described herein are also useful for promoting successful allogeneic or xenogeneic organ and cellular graft transplantation into human patients. In addition, the methods and compositions according to the invention are useful for promoting studies of xenogeneic organ and cellular graft transplants in non-human primates. The patents and publications cited herein illustrate the knowledge available to those skilled in this field and are hereby incorporated by reference in their entirety. Any conflict between these cited references and this specification shall be resolved in favor of this specification.

In a first aspect, the invention provides immunotoxins comprising a cell-specific antigen binding moiety, together with a backbone or carrier moiety, and a down-modulator in an operable linkage. Cell-specific antigen-binding

moiety immunotoxins according to this aspect of the invention comprise the structure:

$$(AB)_n - Y$$

wherein AB represents a cell-specific antigen binding moiety plus a backbone or carrier, Y represents a down-modulatory functional moiety, n is a number from 1 to 2, and – represents an operable linkage, provided, however, that when AB is directed against the CD19, CD38, CD22 or CD7 antigen, then Y is not saporin, that when the AB is directed against CD19, Y is not deglycosylated ricin A chain, and that when the AB is directed against CD22, then Y is not *Pseudomonas* exotoxin A.

For purposes of this aspect of the invention, the following terms are intended to have the meanings set forth below, unless another meaning is specifically set forth. An "antigen-binding moiety", also designated as "AB", is a molecule or macromolecule which binds under physiological conditions to a specific target antigen thereby directing the reduction or elimination of specific plasma cells and lymphoid cells responsible for the production of natural antibodies responsible for graft rejection. AB optionally includes a backbone or carrier, which may be synthetic or naturally derived.

The term "binds under physiological conditions" means forming a covalent or non-covalent association with an affinity of at least 10^9 M^{-1}, either in the body, or under conditions that approximate physiological conditions with respect to osmolarity. For humans, the osmolarity of plasma is about 290 mOsm/kg. As a practical matter, such binding in the body may be inferred either directly from a reduction of specific plasma cells or lymphoid cells responsible for the production of antibodies responsible for allograft rejection or of xenogeneic natural antibodies (XNA) or by a reduction in the antibody titer, or indirectly from an improved survival of a transplanted allogeneic or xenogeneic organ graft. "Cell specific target antigen" or "epitope" are used interchangeably to mean a three-dimensional molecular shape which is bound under physiological conditions by an antigen-binding moiety. In preferred embodiments, such cell specific target

antigens or epitopes are associated with plasma cells and lymphoid cells responsible for the production of antibodies as the basis of allograft rejection or of XNA. More preferably, the cell specific target antigens or epitopes of the invention are found on the surface of the specialized cells responsible for the production of antibodies as the basis of allograft rejection, or of XNA (e.g., on the surface of plasma and lymphoid cells).

In certain preferred embodiments, such antigen binding moiety comprises a complementarity determining region of an antibody which binds under physiological conditions to a peptide-containing special target antigen, or a peptidomimetic of such a complementarity determining region. For purposes of the invention, a "complementarity determining region of an antibody" is that portion of an antibody which binds under physiological conditions to a special target antigen, including any framework regions necessary for such binding, and which is preferably comprised of a subset of amino acid residues encoded by the human heavy chain V, D, and J regions, the human light chain V and J regions, and/or combinations thereof. Examples of such preferred embodiments include an antibody, or an antibody derivative, which may be a polyclonal antibody or more preferably a monoclonal antibody, a chimeric antibody, or an antigen binding antibody fragment.

In addition, given the antibody disclosed herein, those skilled in the art are enabled to make any derivative of such an antibody. For example, Jones et al., (1986) Nature 321: 522 discloses replacing the CDRs of a human antibody with those from a mouse antibody. Marx, (1985) Science 229: 455-456 discusses chimeric antibodies having mouse variable regions and human constant regions. Rodwell, (1989) Nature 342: 99-100 discusses lower molecular weight recognition elements derived from antibody CDR information. Clackson, (1991) BR. Rheumatol. 3952:36-39 discusses genetically engineered monoclonal antibodies, including Fv fragment derivatives, single chain antibodies, fusion proteins, chimeric antibodies, and humanized rodent antibodies. Reichman et al., (1988) Nature 332: 323-327 discloses a human antibody on which rat hypervariable

regions have been grafted. Verhoeyen, et al., (1988) Science 239: 15341536 teaches grafting of a mouse antigen binding site onto a human antibody.

In addition, given the antibody disclosed herein, those skilled in the art are enabled to design and produce peptidomimetics having binding characteristics similar or superior to such complementarity determining region (see e.g., Horwell et al., (1996) Bioorg. Med. Chem. 4: 1573; Liskamp et al., (1994) Recl. Trav. Chim. PhysBas 1:113; Gante et al., (1994) Angew. Chem. Int. Ed. Engl. 33: 1699; Seebach et al., (1996) Helv. Chim. Acta 79: 913). Accordingly, all such antibody derivatives and peptidomimetics thereof are contemplated to be within the scope of the present invention. Compositions according to the invention may further include physiologically acceptable diluents, stabilizing agents, localizing agents or buffers.

In certain preferred embodiments, AB is directed against a plasma cell specific target antigen. Examples of plasma cell specific target antigens according to the invention include, without limitation, CD138 (Syndecan1), CD38, PCA1, CD5, CD19, CD22, CD11b, VLA5, VLA4 and the HMI.24 antigens. In more preferred embodiments of the invention, AB is directed against the CD38, or the HM1.24 antigens, or the Syndecan-1 antigens.

In other preferred embodiments, AB is directed against lymphoid cell specific target antigens. Examples of B cell specific target antigens according to the invention include without limitation CD19, CD20, CD21, CD22, CD23, CD5 and cell surface IgM antigens. In more preferred embodiments, AB is directed against the CD5, CD22, or the cell surface IgM antigens.

For the purposes of the invention, Y may be a chemical or a biochemical agent which is cytotoxic to or capable of down-modulating a target cell. For the purposes of the invention, an agent is cytotoxic or down-modulating if it is capable of impairing a target cell functions. As a practical matter, such cytotoxicity or down-modulation may be inferred either directly from the reduction or elimination of specific cells responsible for the production of antibodies which cause graft rejection (e.g., plasma cells or lymphoid cells) or by a reduction in antibody titer, or indirectly from an improved survival of a

transplanted cell or tissue.

In certain preferred embodiments of this aspect of the invention, AB comprises a backbone or carrier having a molecular weight of from about 5,000 Da to about 1,000,000 Da. In certain other preferred embodiments, Y is an agent having a molecular weight from about 5,000 Da to about 1,000,000 Da.

Preferred agents, Y, according to this aspect of the invention include without limitation, a sphingosine derivative, a lipophilic toxin, a radioisotope, diphtheria toxin, plant toxin, ribosome-inactivating protein, alkaloids, methotrexate, *Pseudomonas* exotoxin A, anti-IgM, and doxorubicin. In a more preferred embodiment, Y is a ribosome-inactivating protein such as saporin, or pokeweed antiviral protein, and in a most preferred embodiment, Y is ricin A chain, provided, however, that when the antigen-binding moiety AB is directed against the CD19, CDA or CD7 antigen, Y is not saporin, that when the antigen binding moiety AB is directed against CD19, Y is not deglycosylated ricin A, and that when the antigen-binding moiety AB is directed against CD22, Y is not *Pseudomonas* exotoxin A. In another most preferred embodiment, Y is a lipophilic toxin selected from N,N-dimethylsphingosine (DMS), N,N,N-trimethyl-sphingosine (TMS), and ceramide analogs. In yet another most preferred embodiment of the invention, Y is selected from a radioisotope, including, but not limited to, ^{131}I, ^{90}Yt, and Tc.

Compositions according to this aspect of the invention may also include pharmaceutically acceptable carriers, diluents, and/or controlled release agents. Certain nonlimiting examples of such carriers, diluents and/or controlled release agents include buffered saline, oils, implantable pumps and encapsulated beads.

"Operable linkage" means any association between AB and Y which allows AB to target plasma cells or B cells which produce antibodies that cause graft rejection which are specific for a specific target antigen or cells which will differentiate to produce antibodies that cause graft rejection which are specific for a specific target antigen. One preferred operable linkage is a covalent linkage directly between AB and Y. Linkages of this type may be formed by activating AB

and coupling the activated AB to an appropriate functional group on Y. Alternatively, AB can be indirectly covalently linked to Y through coupling of both AB and Y to a carrier molecule. For this embodiment, preferred carrier molecules include without limitation neoglycoproteins, such as bovine serum albumin or human serum albumin, other glycoproteins, such as immunoglobulins, interleukins, B cell receptor binding molecules and peptides derived from such neoglycoproteins or glycoproteins, as well as glycolipids, such as HDPE , autologous IgG and synthetic carriers, such as polyglycomers. The compositions of the invention, comprising either direct or indirect types of covalent linkages between AB and Y, can be obtained by procedures analogous to those described in Thall, WO 98/33528, the contents of which is expressly incorporated herein by reference.

Other preferred operable linkages include lipophilic association, such as formation of a liposome containing Y and having AB covalently linked to a lipophilic molecule and thus associated with the liposome. Such lipophilic molecules include without limitation phosphotidylcholine, cholesterol and phosphatidylethanolamine, and synthetic neoglycolipids, such as Galα1,3GalHDPE. In certain preferred embodiments, the operable association may not be a physical association, but simply a simultaneous existence in the body, for example, when AB is associated with one liposome and Y is associated with another liposome. For inducing apoptosis in cells to which AB associates, any of the down-modulatory compositions according to this aspect may further be operably linked to a cell receptor binding peptide or protein.

In certain preferred embodiments, the down-modulatory immunotoxin composition further comprises an inhibitor of protein kinase C (PKC) and/or a member of the Bcl-2 family to promote down-modulation when PKC is operational. . Preferably, the PKC or Bcl-2 family member inhibitor is a sphingosine. More preferably, the sphingosine is N,N-dimethylsphingosine or N,N,N-trimethylsphingosine (TMS). In certain other preferred embodiments, the down-modulatory immunotoxin composition further comprises a small molecule

inhibitor of B cell activation. Preferably, the small molecule inhibitor inhibits *syk-*, *lyn-*, *Btk-*, *blk-*, *fyn-*, and/or *shc*-mediated activation of signal transduction.

The invention also provides a method for facilitating in a transplant recipient of a xenogeneic or allogeneic organ a reduction or down-modulation of those cells responsible for allograft or xenograft antibody mediated graft rejection. The method according to this aspect of the invention comprises administering to the transplant recipient a down-modulatory amount of a down-modulatory immunotoxin composition comprising the structure

$$(AB)_n - Y$$

wherein AB represents a cell-specific antigen binding moiety plus a backbone or carrier, Y represents a down-modulatory functional moiety, n is a number from 1 to 2, and – represents an operable linkage.

Such administration may be via the oral, intravenous, intramuscular, subcutaneous, intra nasal, intradermal, or suppository routes, or by implanting. For initial administration, dosage will depend in part on the size and antigen binding affinity of the particular down-modulatory composition used. Generally, initial dosing will preferably be in the range of about 0.1 to 10 g per 25 kg body weight, and most preferably about 1 g/25 kg. The dosage regimen may be adjusted based upon the response achieved in a particular subject individual. For example, in certain cases a single injection might be sufficient to reduce or eliminate the natural antibody producing cells. In other cases, several divided doses may be administered daily or the dose may be proportionally reduced as onset of down-modulation is observed. Residual specific target antigen cells can be eliminated by administration of chemotherapeutic agents, as described hereinafter. Down modulation is preferably monitored by standard immunoassays for the presence of antibodies specific for the specific target antigen. Thus, for example, when the specific target antigen is Galα1,3Gal, an ELISPOT assay is performed to determine the frequency of αGal secreting B cells. As used herein, "down-modulation" refers to a reduction in the number of cells

expressing the specific target antigen to a level that is lower than it would have been had the method according to the invention not been employed. As discussed above, such down-modulation can be measured, for example, by quantitating a reduction in antibodies specific for Galα1,3Gal epitope. Preferably, such down-modulation will lead to a reduction in such antibodies by 75% or more, preferably 90% or more, and most preferably by 99% to 100%.

In other embodiments a plasma cell directed $(AB)_n$-Y is combined with a B cell directed $(AB)_n$-Y to inhibit antibody production in the transplant recipient. This has the effect of inducing hyporesponsiveness towards cells and tissues of the donor.

In certain preferred embodiments according to this aspect of the invention, the method further comprises administering an inhibitor of protein kinase C (PKC) or an inhibitor of a member of the Bcl-2 family. The PKC inhibitor or inhibitor of a Bcl-2 family member may be administered simultaneously with the $(AB)_n$–Y immunotoxin or may be administered separately. Preferably, the PKC or Bcl-2 family member inhibitor is a sphingosine. More preferably, the sphingosine is N,N,-dimethylsphingosine or N,N,N-trimethylsphingosine (TMS).

In certain other preferred embodiments according to this aspect of the invention, the method further comprises administering, either simultaneously or separately, a small molecule inhibitor of B cell activation. Preferably, the inhibitor of B cell activation inhibits *syk-*, *lyn-*, *Btk-*, *blk-*, *fyn-*, and/or *shc*-mediated activation of signal transduction.

In certain other preferred embodiments, the method further includes inactivating T cells of the recipient mammal by introducing into the recipient mammal an antibody capable of binding to T cells of the recipient mammal.

In still other preferred embodiments, the method further includes inactivating the natural killer cells of the recipient mammal, e.g., by introducing into the recipient mammal an antibody capable of binding to natural killer cells of the recipient mammal prior to introducing the cells or a graft into the recipient mammal. One source of anti-NK antibody is anti-human thymocyte polyclonal

anti-serum. As is discussed below, preferably, a second anti-mature T cell antibody can be administered as well, which lyses T cells as well as NK cells. Lysing T cells is advantageous for both bone marrow and xenograft survival. Anti-T cell antibodies are present, along with anti-NK antibodies, in anti-thymocyte anti-serum. Repeated doses of anti-NK or anti-T cell antibody may be preferable. Monoclonal preparations can be used in the methods of the invention.

In certain preferred embodiments, a short course of an immunosuppressive agent can be administered to inhibit T cell activity in the recipient. In particular, the methods described in U.S. Patent Number 5,876,708 (U.S. Serial Number, filed June 1, 1995), the contents of which are expressly incorporated herein by reference, can be combined with the methods described herein.

In certain other preferred embodiments, the compositions and methods described herein may be used in combination with other compositions and methods for inducing tolerance. In particular, the compositions described herein may be used in combination with the tolerogenic compositions described in Thall, WO 98/33528, the contents of which are hereby expressly incorporated by reference in their entirety.

Methods of inducing down–modulation of antibody-producing cells by the methods described herein can also be combined with other methods for inducing tolerance, e.g., methods which use the implantation of donor stem cells to induce tolerance, e.g., the methods described in U.S. Serial No. 08/451,210, filed on May 26, 1995, the contents of which are hereby expressly incorporated by reference; methods which use stem cells or other tissue from genetically engineered swine, e.g., the genetically engineered swine in U.S. Serial No. 08/292,565, filed August 19, 1994, the contents of which are expressly incorporated herein by reference, or in U.S. Serial No. 08/692,843, filed August 2, 1996, the contents of which are expressly incorporated herein by reference; methods which use the implantation of a xenogeneic thymic graft to induce tolerance, e.g., the methods described in US 5,658,564 (U.S. Serial No. 08/163,912, filed on December 7, 1993), the contents of

which are hereby expressly incorporated by reference; methods of preventing GVHD, e.g., the methods described in U.S. Serial No. 08/461,693, filed June 5, 1995, the contents of which are hereby expressly incorporated by reference; or methods of detecting the presence of swine retroviral sequences, e.g., the methods described in U.S. Serial No. 08/572,645, filed December 14, 1995, the contents of which are hereby expressly incorporated by reference.

The methods described herein can also be combined with the methods of inducing tolerance described in U.S. patent number 5,614,187 (U.S. Serial Number 08/266,427, filed June 27, 1994), the contents of which are hereby expressly incorporated by reference. Thus, the methods disclosed herein can include administering to the recipient a recipient cell which expresses a donor MHC class I gene or a donor MIIC class II gene (or both).

Methods of inducing tolerance by the methods described herein can also be combined with yet other methods for inducing tolerance, e.g., methods which use the implantation of donor stem cells to induce tolerance, e.g., the methods described in U.S. Serial No. 08/451,210, filed on May 26, 1995, the contents of which are hereby expressly incorporated by reference; methods which use stem cells or other tissue from genetically engineered swine, e.g., the genetically engineered swine in U.S. Serial No. 08/292,565, filed August 19, 1994, the contents of which are expressly incorporated herein by reference, or in U.S. Serial No. 08/692,843, filed August 2, 1996, the contents of which are expressly incorporated herein by reference; methods which use the implantation of a xenogeneic thymic graft to induce tolerance, e.g., the methods described in U.S. Serial No. 08/163,912, filed on December 7, 1993, the contents of which are hereby expressly incorporated by reference; methods of increasing the level of the activity of a tolerance promoting or GVHD inhibiting cytokine or decreasing the level of activity of a tolerance inhibiting or GVHD promoting cytokine, e.g., the methods described in U.S. Serial No. 08/114,072, filed August 30, 1993, the contents of which are hereby expressly incorporated by reference; methods of using cord blood cells to induce tolerance, e.g., the methods described in U.S. Serial No.

08/150,739 filed November 10, 1993, the contents of which are hereby expressly incorporated by reference; methods of preventing GVHD, e.g., the methods described in U.S. Serial No. 08/461,693, filed June 5, 1995, the contents of which are hereby expressly incorporated by reference; with methods of promoting tolerance by enhancing or maintaining thymus function, e.g., the methods described in U.S. Serial No. 08/297,291, filed August 26, 1994, the contents of which are hereby expressly incorporated by reference; methods of detecting the presence of swine retroviral sequences, e.g., the methods described in U.S. Serial No. 08/572,645, filed December 14, 1995, the contents of which are hereby expressly incorporated by reference; and the methods for inducing tolerance disclosed in Sykes and Sachs, PCT/US94/01616, filed February 14, 1994, the contents of which are hereby expressly incorporated by reference.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature.

Example 1

Development of an ELISPOT assay to determine the frequency of anti-Gal secreting B cells

In order to determine how experimental manipulations aimed at tolerance induction affect production of anti-Gal, an ELISPOT assay was developed to detect anti-Gal antibody production by B cells in an overnight culture in *vitro*. This assay was based on the detection of binding of antibodies secreted by individual cells to synthetic neoglycoprotein-coated microwells. The method essentially involved the isolation of lymphocytes or lymphocyte preparations enriched for B cells from various tissues. These cells were incubated in microwells in a hybridoma culture medium containing insulin, transferrin, and selenium, with or without fetal calf serum. In some cases, cells were immediately transferred to nitrocellulose bottom microtiter wells coated with antigen. Alternatively, cells were allowed to incubate with different cytokines and/or

antibodies for up to three days before adding them to the antigen coated microwells. After an overnight incubation in the antigen coated microwells, cells were washed from the wells and goat anti-mouse IgM or IgG conjugated to horseradish peroxidase was added. After incubation and removal of the secondary reagent, a peroxidase substrate was added. Spot formation was then taken to represent clonal secretion of antigen specific antibody. Each antigen specific clone is represented as a spot forming unit (SFU). The frequency of cells secreting antibody is then calculated as the number per 10^5 cells added to the microwells. The GalT(-/-) mouse was used throughout these studies as a model for the Galα1,3Gal specific XNA in a murine model. It is an embryonic stem cell knockout of the Galactosyltransferase gene. The result is a mouse which produces Galα1,3Gal specific XNA.

Specifically, the methodology was as follows:

A microtiter plate (Millipore 96-well filtration plate, 0.45 um surfactant-free mixed cellulose ester membrane, Qty: 10/pack, Cat# MAHAS4510) was aseptically coated with 100µl/well of 5ug/ml αGal-BSA in 1xPBS, and/or 100µl/well of unlabeled IgM and IgG at 5ug/ml in 1xPBS. Appropriate controls, such as N-acetyllactosamine-BSA were included. The coated plates were incubated overnight at 4°C, or 2 hours at 37°C. After incubation, the antigen solution was aseptically pipetted out of the plates. The plates were then washed by pipetting 200µl/well of 1xPBS, sitting for 5 minutes and then pipetting out of the solution. The wells were washed two times with 200µl/well of PBS and pipetted with 1xPBS up and down. Next, the microtiter wells were blocked for non-specific antibody binding with 200µl/well of IMDM (Iscove's Modified Dulbecco's Medium) supplemented with 0.4% BSA and 1ml/500ml of Gentamicin and incubated for one hour at 37°C. The blocking medium was removed by pipetting it out. Then, 200µl/well of cell culture medium was added to rows B-D and rows F-H. The cell culturing medium was made with IMDM supplemented with 10% Fetal Bovine Serum. Then, 250µl/well of the spleen cell preparation with a concentration of $4x10^6$ cells per ml was added to each well. The cells were serially

diluted by 1/5 by taking 50µl from row A and transferring it to row B and then removing 50µl from row B and transferring it to row C and the same for row C to row D. 50µl was removed from row D and discarded to have 200µl in each well. This dilution was repeated with rows E through H. The plates were incubated overnight at 37°C, with 5% CO_2. Following incubation, the plates were emptied by dumping and "flicking" out. The plates were washed three times with 200µl/well of 1xPBS. For the first two washes, 1xPBS was pipetted up and down to get rid of cells stuck to the membrane and to reduce the background. Then the plates were washed three times with 200µl/well of 1xPBS + 0.1% Tween-20. Next, 10µl/well of HRP-conjugated anti-mouse IgG or IgM was added and the wells were diluted to 1/1000 in 1xPBS supplemented with 0.5% Tween, and 0.4% BSA. The plates were then emptied out by dumping and "flicking" out the solution. The plates were washed three times with 200µl/well of 1xPBS + 0.1% Tween-20 followed by three washes with 200µl/well of 1xPBS. The substrate solution was added at 100µl/well and incubated at room temperature for 30 minutes. The substrate was made by dissolving 1 AEC (3-amino-9-ethylcarbazole) tablet (Sigma A-6926) in 2.5ml dimethylformamide acetate. After it has dissolved, 47.5ml of 50mM Acetate buffer, pH 5.0 (74ml of 0.2N acetic acid, and 176ml 0.2M sodium acetate, then deionized water up to 1000ml) was added. 25µl of fresh 3% H_2O_2 was added just before adding the substrate solution to the plate. The reaction was stopped by running tap water over the plate and dumping/flicking the water in the wells into the sink, and blotting dry. The bottom plastic section of the plate was removed and placed on C fold towels. The plate was wrapped in aluminum foil and allowed to dry at room temperature for 1-2 days. Spots were visualized with a stereomicroscope and dissecting with vertical white light. The spots were counted as the number of dark centers with rings of diffusion per well. The wells were averaged in triplicate, and divided by 8 to get SFUs (Spot Forming Units) per 10^5 cells. For example,

$4.0x10^6$ cells/ml x 0.2ml/well = $8.0x10^5$ cells/well

Diluted by 1/5 = $1.6x10^5$ cells/well

Diluted by 1/5 = $3.2x10^4$ cells/well

Diluted by 1/5 = $6.4x10^3$ cells/well

The specificity of the anti-Gal ELISPOT assay for Galα1,3Gal epitopes was demonstrated by incubating either GalT(-/-) spleen cells or frozen baboon spleen cells overnight in plates coated with terminal Galα1,3Gal, Lacto-N-fucopentaose (LNFP) or N-Acetyllactosamine (LacNAc) conjugated to BSA. Specific spot formation was only significantly above background when baboon spleen cells were cultured on Galαl,3Gal-linked neoglycoprotein (Figure 1). The specificity of the anti-Gal ELISPOT analysis using Ga1T(-/-) mouse spleen cells was also shown in an analogous manner to that of the baboon spleen ELISPOT data (Figure 2).

The results of these experiments indicated that this ELISPOT assay is highly specific for the detection of the frequency of anti-Gal secreting plasma cells. Therefore, this ELISPOT assay has been used as the basis for the determination of the state of anti-Gal production by B cells in many subsequent experiments.

Example 2

The effect of treatment of GalT(-/-) mice with anti-IgM $F(AB')_2$ on antibody production

GalT (-/-) mice were injected i.p. with either goat anti-mouse IgM $F(ab')_2$, goat anti-human IgM $F(ab')_2$ or PBS. Antibodies were injected on day 0 at 30mg/kg, followed on days 1,2, and 3 at 15mg/kg/day. On day 4, lymphocytes were isolated from the spleen and subjected to anti-Gal and total IgM ELISPOT analysis. (Figures 3-4)

These data provided evidence that antigen receptor cross-linking is sufficient to abrogate antibody production. Total IgG production was higher after injection with the control goat anti-mouse IgM preparation than the PBS injection control. This indicated that goat antibodies are capable of stimulating an IgG response, which is thought to involve the cooperation of T cells for mediating a class switch from IgM to IgG. This clearly indicated that a molecule which can both cross-link the cell surface antigen receptor and stimulate T cell dependent antibody class

switching is capable of reducing antibody production. This treatment method may be used in combination with the B cell tolerance method as set forth herein.

Example 3

In vitro inhibition of baboon anti-Galα1,3Gal antibody production using anti-CD38 immunotoxin

This Example describes a method, using an immunotoxin, that results in the inhibition of the production of anti-Galα1,3Gal antibodies in an *in vitro* setting. Due to the paucity of knowledge regarding the phenotype of the plasma cells which produce anti-Galα1,3Gal antibodies the immunotoxin used in this Example would be expected to result in the elimination of the majority of plasma cells. Specifically, the immunotoxin comprised an anti-CD38 antibody (OKT10, J& J) conjugated to ricin A chain (RCA).

Anti-CD38 antibody was reacted with sulfo-LeSPDP (sulfosuccinimidyl 6[3'(pyridyldithio)propionamido] hexanoate) at a molar ratio of 1:8. The modified antibody was separated from free SPDP by chromatography on Sephadex G-25 column. Deglycosylated ricin A chain (dRCA) was reduced by dithiothreitol (DTT) and separated from free dithiothreitol (DTT) on G-25. The SPDP modified antibody was then reacted with the reduced dRCA at a molar ratio of 115 for 24 hours at 20°C. Free dRCA was separated from the conjugate by chromatography on Sephacryl S-100.

Baboon spleen cells then received an immunotoxin treatment with the anti-CD38- dRCA immunotoxin. Freshly isolated baboon spleen cells (10^7 cells) were incubated with anti-CD38-dRCA or a isotype class matched control nonbinding antibody toxin conjugate MOPC31dRCA at 5 (g/ml for 1 hr. at 37°C. Cells were washed and incubated in DMEM medium containing 0.3% BSA, insulin, selenium and transferrin for 48 hours. Cells were plated onto microwells for antiGal ELISPOT analysis (Example 1).

As can be seen in Figure 5 treatment of baboon spleen cells with unconjugated anti-CD38 or the control conjugate MOPC31-dRCA had no effect on the frequency

of cells capable of secreting anti-Gal. However, the anti-CD38-dRCA immunotoxin abrogated anti-Gal production by baboon spleen cells. This suggested that the anti-CD38-RCA immunotoxin can specifically down-modulate anti-Gal production, presumably via a mechanism of internalization of the immunotoxin, followed by cell death.

Example 4

Immunoaffinity adsorption removes XNA but does not prevent the return of XNAs in Animals

Baboons (Papio anubis) weighing 8-15 kg were obtained from Biological Resources, Houston, TX. All procedures involving baboons were carried out in accordance with the NIH Guidelines for Care and Use of Laboratory Animals and were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Surgical Procedures

All surgical procedures using extracorporeal immunoadsorption (EIA) were carried out under inhalation anesthesia consisting of isoflurane, oxygen, and nitrous oxide. Prior to anesthesia, animals received atropine and were sedated with ketamine.

Gal Immunoaffinity Column

Each column (capacity 50 ml) contained 50 g of matrix material composed of 25 mg of Galα1,3Galα1-4Glc(αGal trisaccharide type VI) bound to silica particles (Alberta Research Council, Edmonton, Canada). Previous *in vitro* studies confirmed that 50 g of matrix material coated with 25 mg of synthetic sugar adsorbed all anti-Galα1,3Gal antibody from a maximum of 1,250 ml of plasma (Xu et al., 1998. Transplantation 65:172-179). Each column was primed by perfusion with 600 ml saline containing 100 ml of 5% human albumin and then flushed with 2-3 l of 0.9% saline at a flow rate of 40 ml/min. Finally, the column was flushed with 500 ml of

saline containing 6,000 units of heparin.

Apheresis and Plasma Perfusion through an αGal Immunoaffinity Column (CPA)

This procedure was performed using a COBE-Spectra pheresis unit (Blood Component Technology, Inc., Lakewood, CO) for separation of plasma from cellular components, following a protocol for therapeutic plasma exchange designed for pediatric patients. The total volume of the extracorporeal system (primed with 0.9% saline) was approximately 300 ml. Blood was withdrawn from an external jugular central venous catheter and returned into a saphenous vein. Three plasma volumes were immunoadsorbed on each occasion, the average perfusion rate of plasma passing through the column being 20 ml/min. After passage through the column, the plasma was returned to the baboon. To avoid thrombotic complications during pheresis, anticoagulation was achieved with citrate phosphate dextrose adenine (CPDA) or heparin immediately prior to surgery. Baboons treated with CPDA required calcium supplementation with 10% calcium gluconate (American Reagent Laboratories, Inc., Shirley, NY) 10-14 ml/hr by intravenous infusion.

Transient hypotension was observed with initiation of EIA and was corrected by i.v. crystalloid administration and neosynephrine infusion when systemic arterial pressure was reduced below 80 mmHg.

ELISA for measurement of anti-Galα1,3Gal antibody

A 0.0162% concentration of baboon serum was loaded on a Maxisorb plate (Nunc, Naperville, IL) coated at a concentration of 5 (μl/mL of Galα1,3Gal trisaccharide type VI conjugated to BSA (Alberta Research Council) and incubated for 1 hr at 37°C. Bound antibodies were detected using polyclonal donkey antihuman IgG (Accurate Chemical and Scientific Co., Westbury, NY) or rabbit antihuman IgM (Dako, Copenhagen, Denmark) conjugated to horseradish peroxidase. Plates were washed and color development was achieved by using o-phenylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, MO) as a

substrate at 0.9 mg/ml in a phosphate citrate buffer with urea hydrogen peroxidase (Sigma Chemical Co., St. Louis, MO). Absorbance at 490 nm was determined by a THERMOmax plate reader (Molecular Devices, Palo Alto, CA). All samples were tested in triplicate. For comparison of effectiveness of Galα1,3Gal antibody removal and the pattern of recovery, antibodies were quantified by extrapolation of sample absorbance values against a standard curve generated on every plate using purified baboon Galα1,3Gal IgG and IgM.

ELISA for measurement of total IgG and IgM

Maxisorb plates (Nunc, Inc., Naperville, IL) coated with 100 (μl/well of 5 (μg/mL of polyclonal donkey antihuman-IgG (Accurate Chemical and Scientific Co., Westbury, NY) or 1:3,000 dilution of rabbit antihuman-IgM (Dako, Copenhagen, Denmark) were loaded with 100 (μl/well of serial dilutions of baboon serum and incubated for 1 hr at 37°C. Bound antibodies (IgG or IgM) were detected using horseradish peroxidaseconjugated donkey antihuman IgG (Accurate Chemical and Scientific Co., Westbury, NY) and rabbit antihuman IgM (Dako, Copenhagen, Denmark). After 1 hr of incubation at 37°C, plates were washed and color development achieved using o-phenylenediamine dihydrochloride as a substrate at 0.9 mg/ml in PBS with urea hydrogen peroxidase (Sigma). Development was blocked with 50 (μl of 2 N H_2SO_4. Absorbance at 490 nm was measured as described above. The results of CPA using a representative naïve baboon B75-23 are shown in Figure 6. Most antibody was removed during the immunoadsorption of the first plasma volume. After a single CPA of 3 plasma volumes, a rapid return of antibody to preimmunoadsorption level occurred with 1-4 days (and usually reached approximately 50% of preadsorption level within 2 days). Thus plasma perfusion of baboon blood through a synthetic Galα1,3Gal column is effective in removing anti Galα1,3Gal antibodies. A single CPA in an immunologically naïve baboon had only a temporary effect on reduction of anti- Galα1,3Gal antibody, with a return to preadsorption level within 1-4 days. Multiple sequential CPAs were efficient in depleting the pool of circulating anti- Galα1,3Gal antibody, but only the first three or

four adsorptions provided additional reductions in the level of antibody.

Example 5

Use of anti-human/primate CD38-deglycosylated ricin A chain immunotoxin for depletion of anti-(Gal antibody secretion *in vivo*

Baboon B55-129 was treated with 0.1 mg/kg of the anti-CD38-dRCA immunotoxin for 14 consecutive days (days 0-14). On days 5, 6, and 7 IgG- and IgM-XNA were removed by column perfusion procedures. The baboon received cyclosporine throughout the experiment.

The levels of anti-CD38 in the circulation (Figure 7) over the 14 day regimen varied between 100-180 ng/ml one hour after infusion and 30-80 ng/ml the next day. This baboon developed an IgG- and IgM- anti-mouse antibody titer as early as one day after the last anti-CD38-dRCA immunotoxin dose (Figure 8).

Results obtained using this baboon (Figure 9) demonstrate for the first time that production of XNA reactive with the Galα1,3Gal epitope can be significantly delayed . The three column perfusion procedures on days 5, 6, and 7 completely depleted IgG- and IgM-XNA which remained undetectable for the duration of the immunotoxin treatment. The failure to effect long-term inhibition of XNA production at this time could be due to the recovery of the baboon from the effects of anti-CD38-dRCA immunotoxin.

Example 6

Effect of DMS containing liposomes *in vitro* and *in vivo* in mice on anti-gal production

N,N-dimethylsphingosine (DMS) , a terminal component of the sphingosine metabolic pathway, has been shown by others to inhibit the proliferation of human B cell lines via apoptosis. To determine whether anti-Gal secreting cells from tissues were also susceptible to metabolic inhibition through the sphingosine cell death pathway, these lymphocytes were removed from the spleen of GalT(-/-) mice. These cells were treated with DMS containing liposomes followed by an anti-Gal

specific ELISPOT assay.

The specific methodology was as follows:

Blank liposomes were composed of phosphatidylcholine (PC) and cholesterol (C) in a ratio of 60:40. DMS liposomes consisted of PC:C:DMS in a ratio of 60:40:6. Liposomes were prepared by dissolving compounds in chloroform and drying them under vacuum rotation evaporation, followed by rehydration in PBS, freezing in liquid N_2 and thawing using a 45°C water bath. The freeze-thaw procedure was repeated four times. 100 nm liposomes were formed by extrusion through a 100 nm polycarbonate membrane for 10 times using a LIPEX™ extruder (Lipex Biomembranes, Vancouver, BC). Liposome size was confirmed on a particle size analyzer (Brookhaven Instruments, Holtsville, N.Y.). For *in vivo* experiments, mice were injected with 10 mg liposomes by i.v. injection. Therefore, in the case of DMS liposomes, the equivalent of 600 micrograms of DMS was injected. Figures 10 and 11 show that DMS-containing liposomes completely inhibit *in vitro* and *in vivo* αGal production, respectively. These data demonstrated that liposomes incorporated with glycolipids containing N,N-dimethylsphingosine are capable of down-modulating anti-Gal production.

Example 7

Antigen receptor cross-linking diminishes anti-gal production *in vitro*

dimethylsphingosine (DMS) can down modulate anti-Gal production *in vivo* and *in vitro* (Figures 10 and 11). Anti-IgM $F(ab')_2$ abrogates anti-Gal production *in vitro* in GalT(-/-) mouse spleen cells and in baboon spleen cells (Figure 12).

Therefore, we were interested to combine the anti-IgM $F(ab)'_2$ treatment with DMS. Negative signals through the antigen receptor are thought to involve, in part, the generation of sphingosine metabolites. Therefore, we hypothesized that a potential synergy or additive effect might ensue as a result of simultaneous treatment with both of these agents

These results with baboon spleen cells indicated that it is advantageous to combine antigen receptor cross-linking with treatment with DMS. Figure 13 shows

that the combination of these treatments abrogates anti-Gal production by baboon cells. It is therefore intended that antigen receptor cross-linking with specific down modulators of the anti-Gal cell surface receptor on B cells and plasma cells be combined with a form of metabolic inhibition in which B lineage cells display a pronounced down modulation.

Example 8

Anti-CD22 immunotoxin –mediated inhibition of antibody production by baboon cells

In order to identify potential new anti-B lineage immunotoxins, we conducted an experiment to ascertain whether anti-human CD19 (HD37), anti-CD22 (RFB4) and antibodies (gifts from Ellen Vitetta, The University of Texas Southwestern Medical Center, Dallas 75235-8576) would cross-react with baboon spleen cells. Human and baboon splenocytes were thawed, FICOLL™(-purified, and analyzed by a 2-color indirect flow cytometric screening analysis. Staining of the cells was done in three steps: 1. Incubated 9 x 10^5 cells per sample with the purified anti-CD19, or anti-CD22, and the mouse IgG1 isotype control for the antibodies. Added 5μg of each antibody. 2. Cells were washed, and incubated with goat $F(ab')_2$ anti-mouse IgG1-RPE. 3. Cells were washed, incubated with 1% mouse serum to block unbound goat anti-mouse $F(ab')_2$ and then added anti-CD20 FITC, and mouse IgG1–FITC to the appropriate samples. Mouse IgG1-FITC is the isotype for anti-CD20. The analysis of the samples was to look at the FL-1 versus FL-2 channels of the lymphocyte gate.

Results of the analysis show the dot blot quadrants for the anti-CD22-PE 2" versus CD20-FITC for both human and baboon cells. Figure 14 shows that the anti-human CD22 antibody cross-reacts with baboon cells.

An indirect cytotoxicity assay was performed which involves measuring the inhibition of protein synthesis in baboon cells. Anti-CD22 (RFB4) or anti-TNP as a control, was added at 1x 10^{-10} M final concentration for 45 min at 4°C to 4 x 10^6 cells/ml. Anti-mouse IgG-Fab-dgRCA was added at 0.5 (μg/ml final concentration

and incubated overnight. The cells were washed and cultured for an additional 24 hrs. Serial dilutions were made into ELISPOT plates for analysis of the relative frequencies of total IgG and IgM-secreting cells. Figure 15 shows that the anti-CD22 immunotoxin specifically inhibits anti-gal production by baboon cells.

Example 9

Use of Anti-CD22 dg-Ricin A to deplete baboon antibody secreting cells.

We have determined that CD22 is expressed on activated B cells and antibody secreting B lineage cells. Since baboons bind the monoclonal anti-human CD22, designated RFB4, we examined splenocytes for the effect of anti-CD22 deglycosylated Ricin A chain (dg-Ricin A; dgRCA; dRCA) on total IgM production by ELISPOT analysis. Anti-CD22 dg-Ricin A was incubated with baboon splenocytes, depleted of T cells, for 1hr, followed by washing and overnight incubation. Cells were then added to ELISPOT wells at $4x10^5$ cells/well and incubated overnight. The results, shown in Figure 16, show a specific dose dependent decrease the frequency of IgM secretors when ELISPOTs are enumerated following incubation with the immunotoxin. These data indicate that anti-CD22-dg-Ricin A chain can down-modulate or deplete CD22+ cells producing IgM. Anti-Gal secretors are represented in this total IgM producing population. Therefore, anti-CD22 can be used in conjunction with other anti-B lineage reagents for the down-modulation of antibody production, inclusive of anti-Gal.

What is claimed is:

1. A down-modulatory immunotoxin composition comprising the structure:

$$(AB)_n - Y$$

wherein AB represents a cell-specific antigen binding moiety plus a backbone or carrier, Y represents a down-modulatory functional moiety, n is a number from 1 to 2, and – represents an operable linkage, wherein the antigen-binding moiety AB is directed against a B cell target antigen, provided, however, that when the antigen-binding moiety AB is directed against the CD19, CD38, CD22 or CD7 antigen, then Y is not saporin, that when the antigen-binding moiety AB is directed against CD19, Y is not deglycosylated ricin A, and that when the antigen-binding moiety AB is directed against CD22, then Y is not *Pseudomonas* exotoxin A.

2. The down-modulatory immunotoxin composition according to claim 1, wherein the B-cell target antigen is selected from the group consisting of CD19, CD20, CD21, CD22, CD23, CD5, and cell surface IgM.

3. The down-modulatory immunotoxin composition according to claim 1, wherein the B-cell target antigen is selected from the group consisting of CD5, CD22, and cell surface IgM.

4. A down-modulatory immunotoxin composition comprising the structure:

$$(AB)_n - Y$$

wherein AB represents a cell-specific antigen binding moiety plus a backbone or carrier, Y represents a down-modulatory functional moiety, n is a number from 1 to 2, and – represents an operable linkage, wherein the antigen-binding moiety AB is directed against a plasma cell specific target antigen, provided however, that when

AB is directed against the CD38 antigen, Y is not saporin.

4. The down-modulatory immunotoxin composition according to claim 3, wherein the plasma cell specific target antigen is selected from the group consisting of CD138, CD38, PCA1, CD5, CD19, CD22, CD11b, VLA5, VLA4, and the HM1.24 antigens.

5. The down-modulatory immunotoxin composition according to claim 3, wherein the plasma cell specific target antigen is selected from the group consisting of CD38, CD138, and the HM1.24 antigens.

6. The down-modulatory immunotoxin composition according to claim 1 or 2, wherein the operable linkage is a lipophilic association.

7. The down-modulatory immunotoxin composition according to claim 1 or 2, further comprising an inhibitor of protein kinase C (PKC) or an inhibitor of a member of the Bcl-2 family.

8. The down-modulatory immunotoxin composition according to claim 1 or 2, further comprising an inhibitor of B cell activation.

9. A method for facilitating in a transplant recipient of a xenogeneic or allogeneic organ a reduction or down-modulation of those cells responsible for allograft or xenograft antibody mediated graft rejection, the method comprising the step of administering to the transplant recipient a down-modulatory amount of a down-modulatory immunotoxin composition comprising the structure:

$$(AB)_n - Y$$

wherein AB represents a cell-specific antigen binding moiety plus a backbone or carrier, Y represents a down-modulatory functional moiety, n is a number from 1 to

2, and – represents an operable linkage

10. The method of claim 9, wherein the antigen-binding moiety AB is directed against B cell target antigens.

11. The method of claim 9, wherein the antigen-binding moiety AB is directed against plasma cell target antigens.

12. The method of claim 9, wherein the transplant recipient is a human and the xenograft organ is from a pig.

FIGURE 1

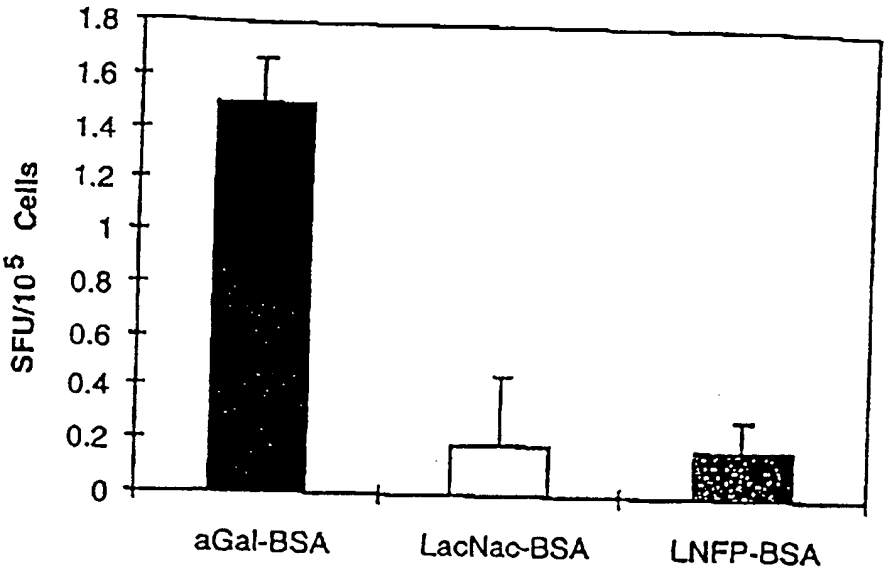

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SFU/10^5 Cells
aGal-BSA
LacNac-BSA
LNFP-BSA

FIGURE 2

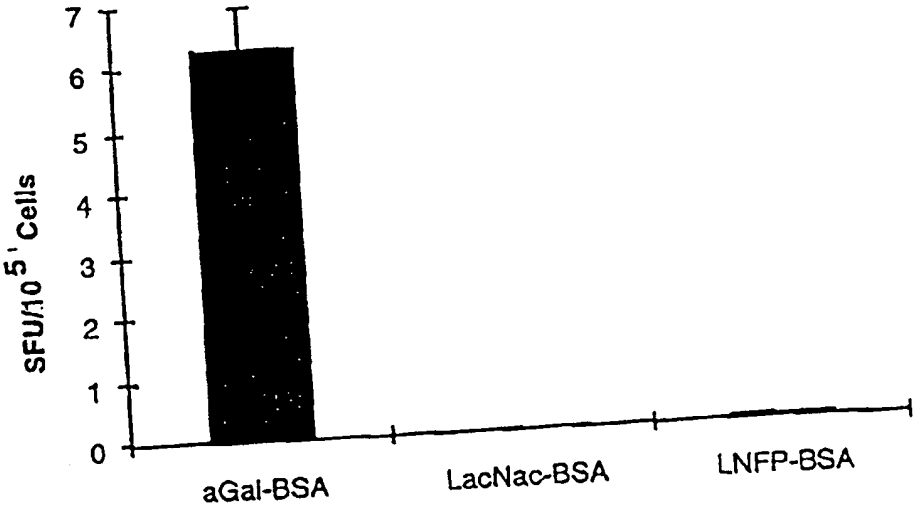


FIGURE 3

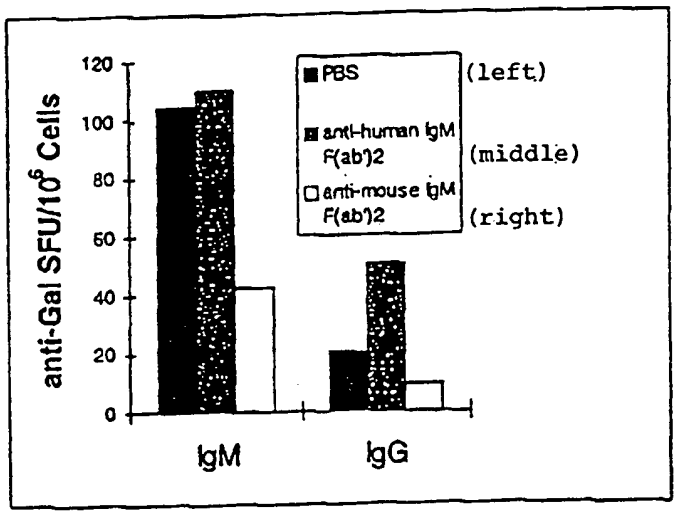

anti-Gal SFU/10^6 Cells
120
100
80
60
40
20
0
PBS
(left)
anti-human IgM F(ab')2
(middle)
anti-mouse IgM F(ab')2
(right)
IgM
IgG

FIGURE 4

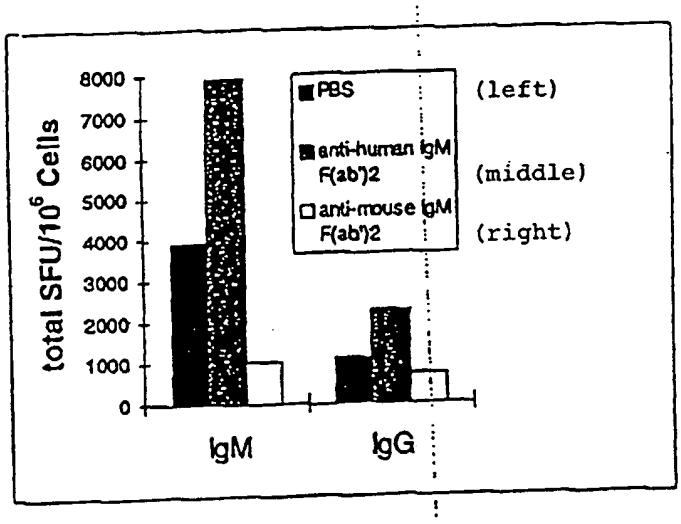

8000
7000
6000
5000
4000
3000
2000
1000
0
total SFU/10^6 Cells
PBS
anti-human IgM F(ab')2
anti-mouse IgM F(ab')2
(left)
(middle)
(right)
IgM
IgG

FIGURE 5

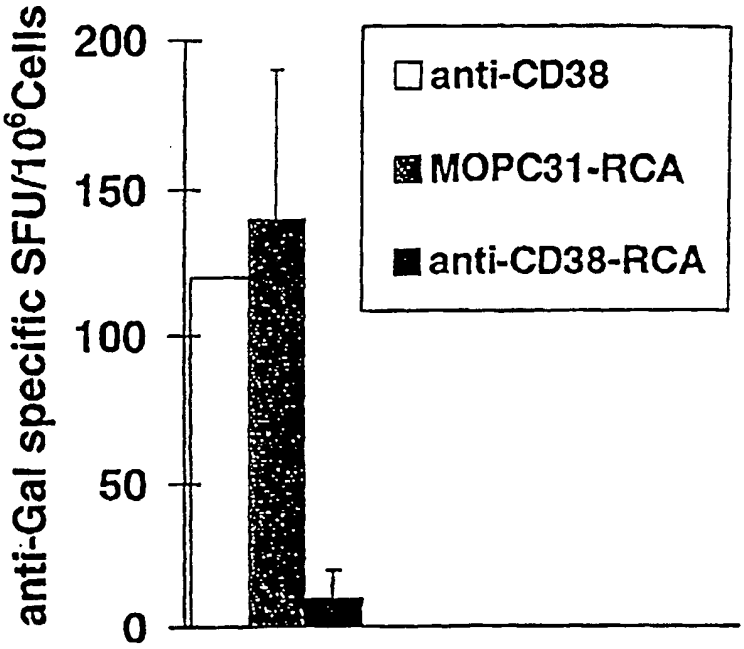

anti-Gal specific SFU/10^6Cells
200
150
100
50
0
anti-CD38
MOPC31-RCA
anti-CD38-RCA

FIGURE 6

XNA Profile of Baboon B75-23, Subjected to Immunoaffinity Adsorption of XNAs

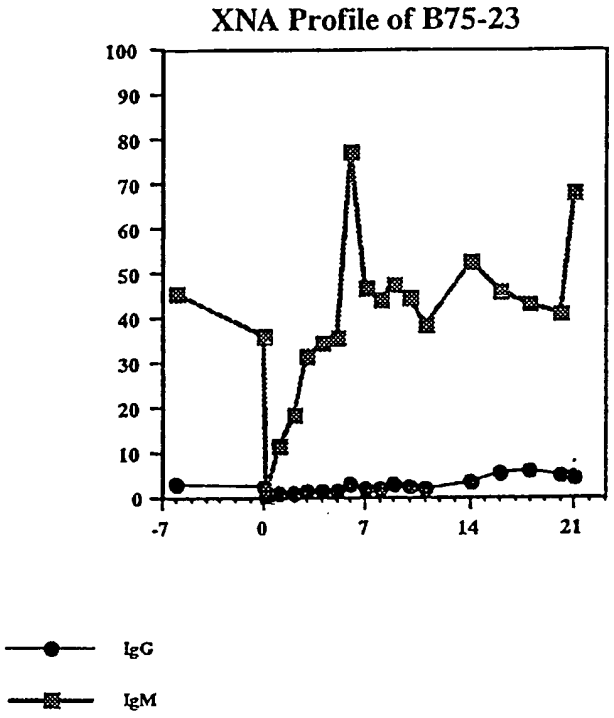


FIGURE 7

Circulating Anti-CD38 Levels in the Baboon Administered Anti-CD38-dRCA Immunotoxin (B55-129)

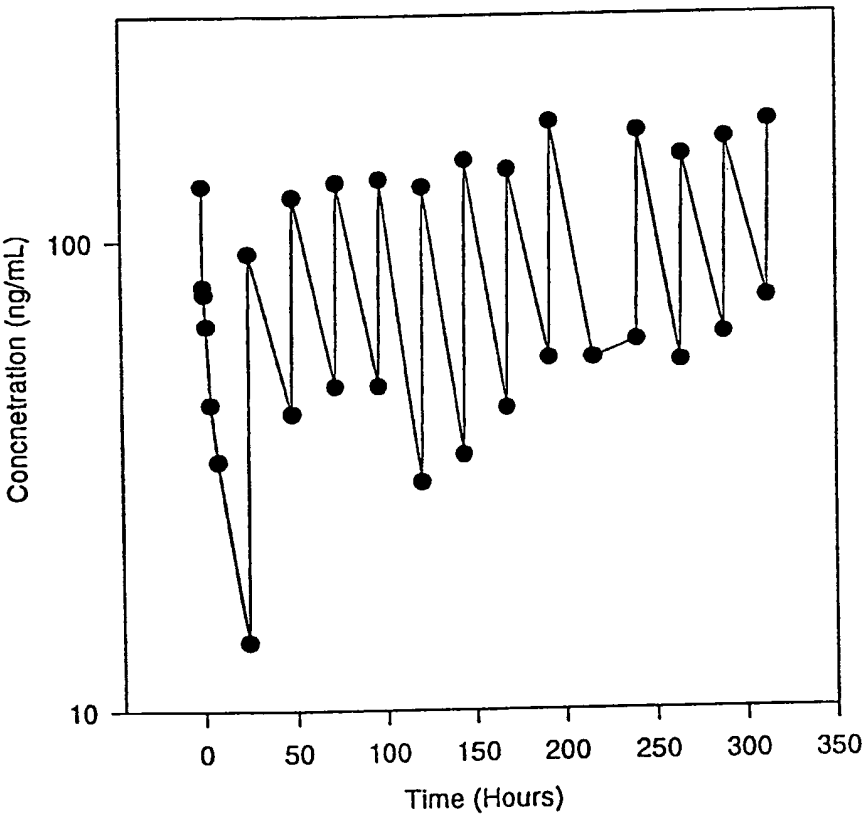


FIGURE 8

Baboon Anti-Mouse Response (BAMA) in the Baboon Administered Anti-CD38 Immunotoxin (B55-129)

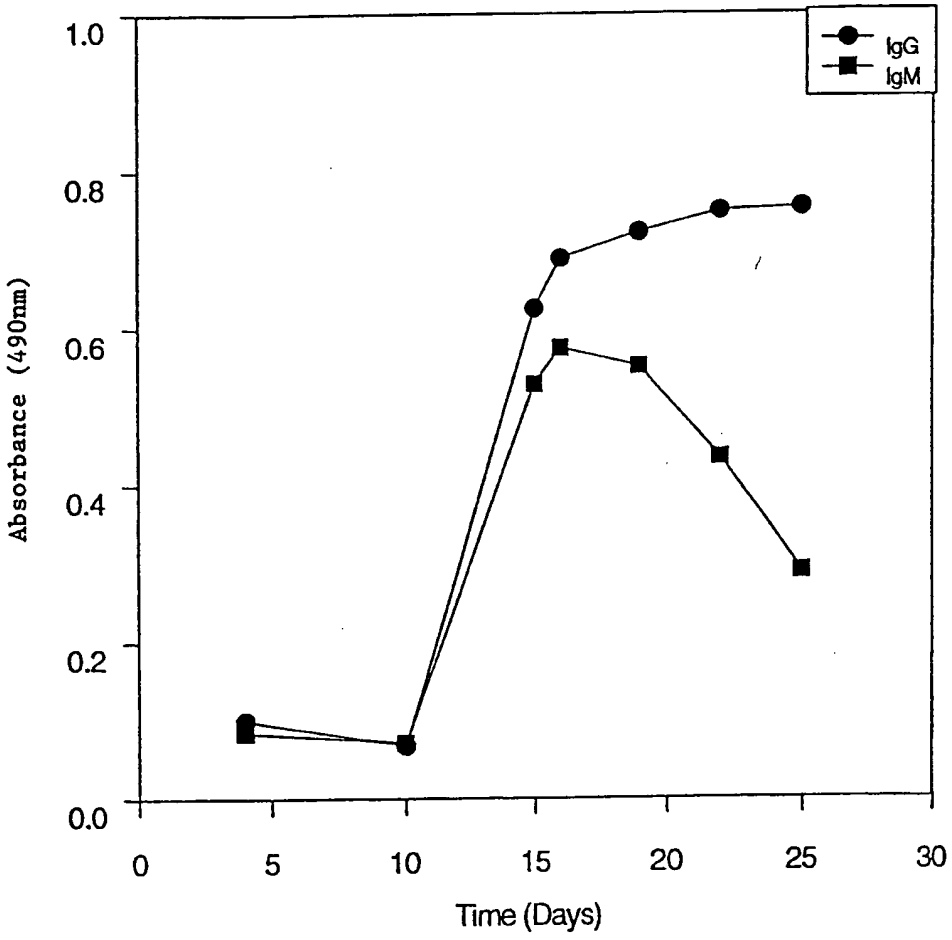


FIGURE 9

Levels of Circulating XNA in the Baboon (B55-129) Administered Anti-CD38 Immunotoxin

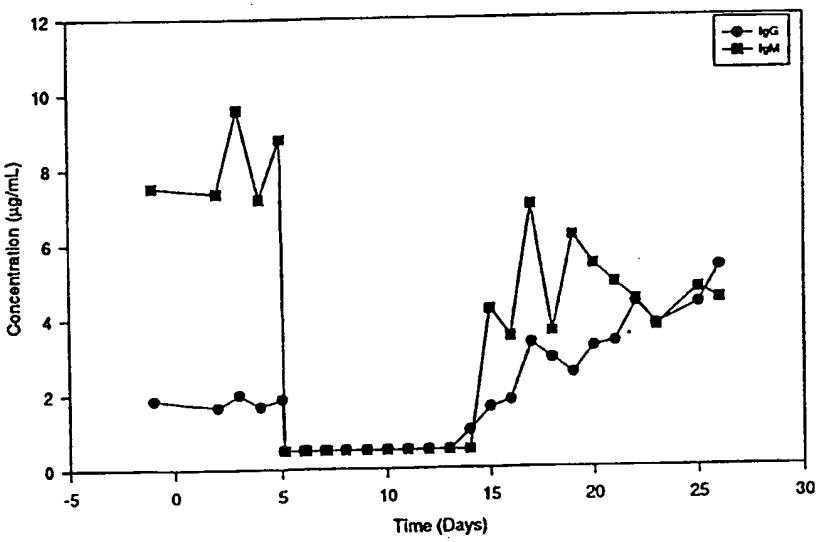


FIGURE 10

DMS containing liposomes completely inhibit *in vitro* anti-Gal production

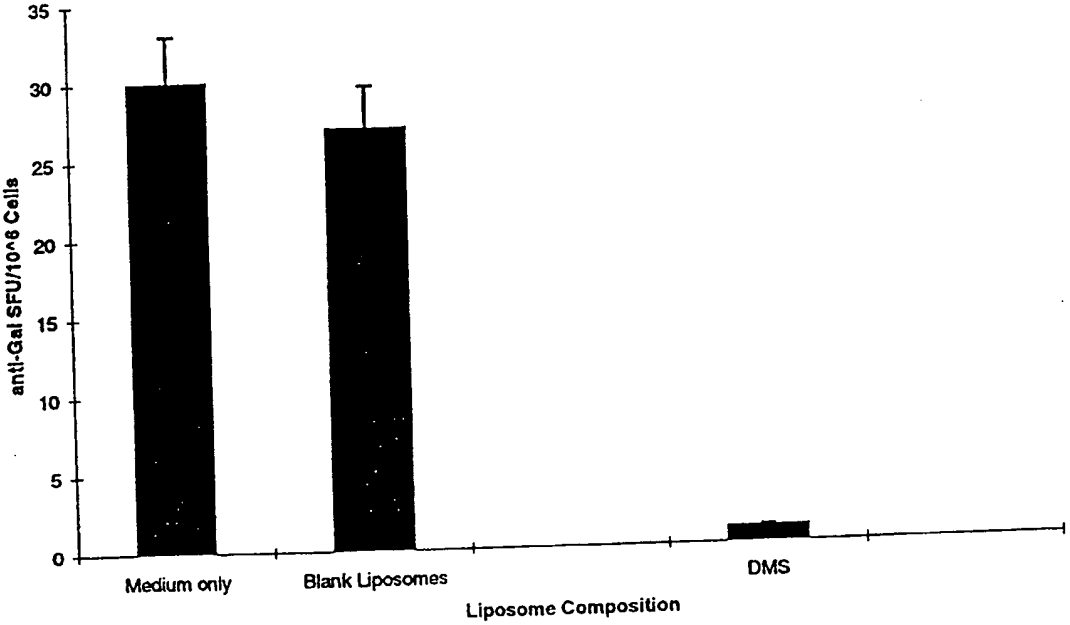


FIGURE 11

DMS containing liposomes significantly decrease the frequency of anti-Gal secreting cells in GalT(-/-) mice.

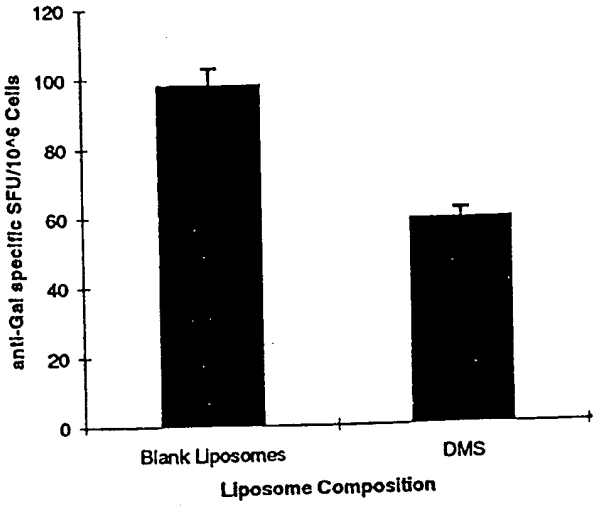


FIGURE 12

Effect of *in vitro* treatment of GalT(-/-) mouse and baboon B36-46 spleen lymphocytes with anti-IgM F(ab')$_2$ on the frequency of anti-Gal IgM and IgG-secreting cells

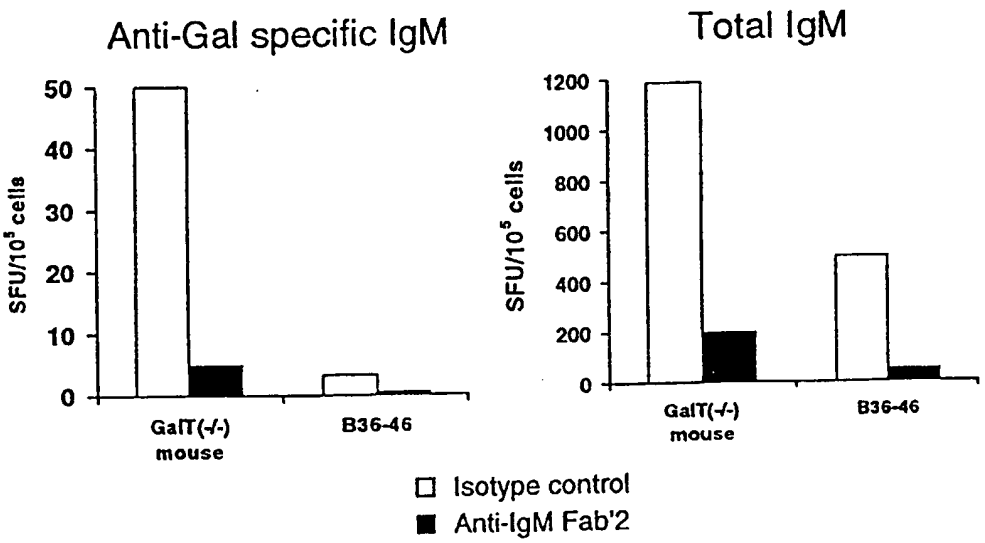


FIGURE 13

Effect of combination of anti-IgM with DMS on IgM anti-Gal production by baboon spleen cells *in vitro*

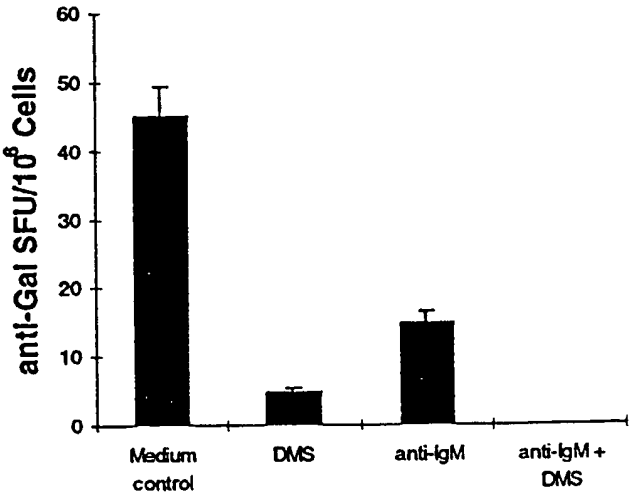


FIGURE 14

Cross-reactivity of anti-CD22 with human or baboon spleen B cells

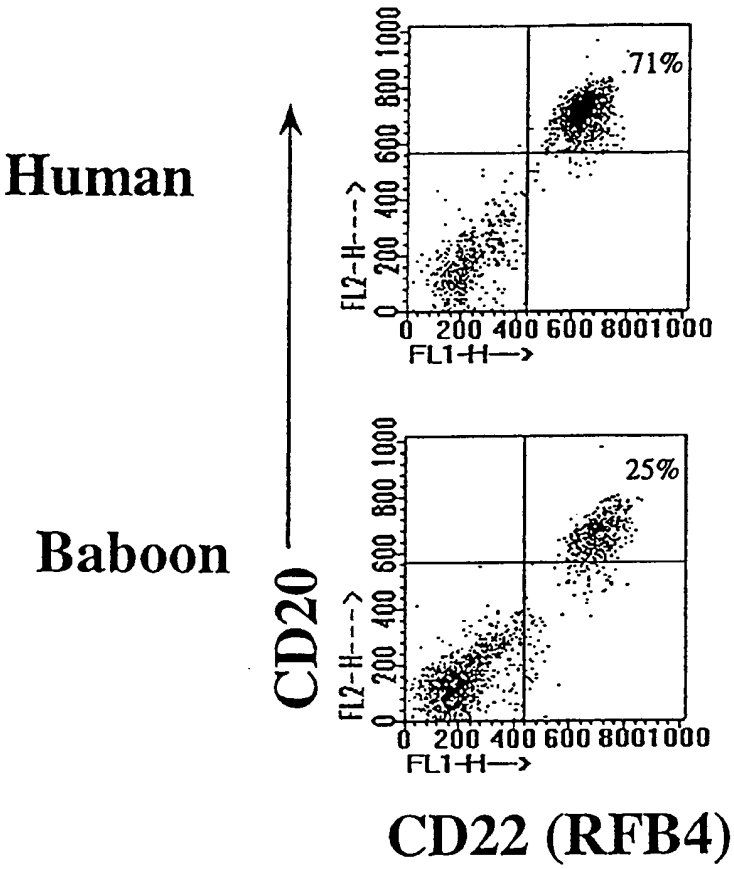


FIGURE 15

Anti-CD22 immunotoxin –mediated inhibition of antibody production by baboon cells

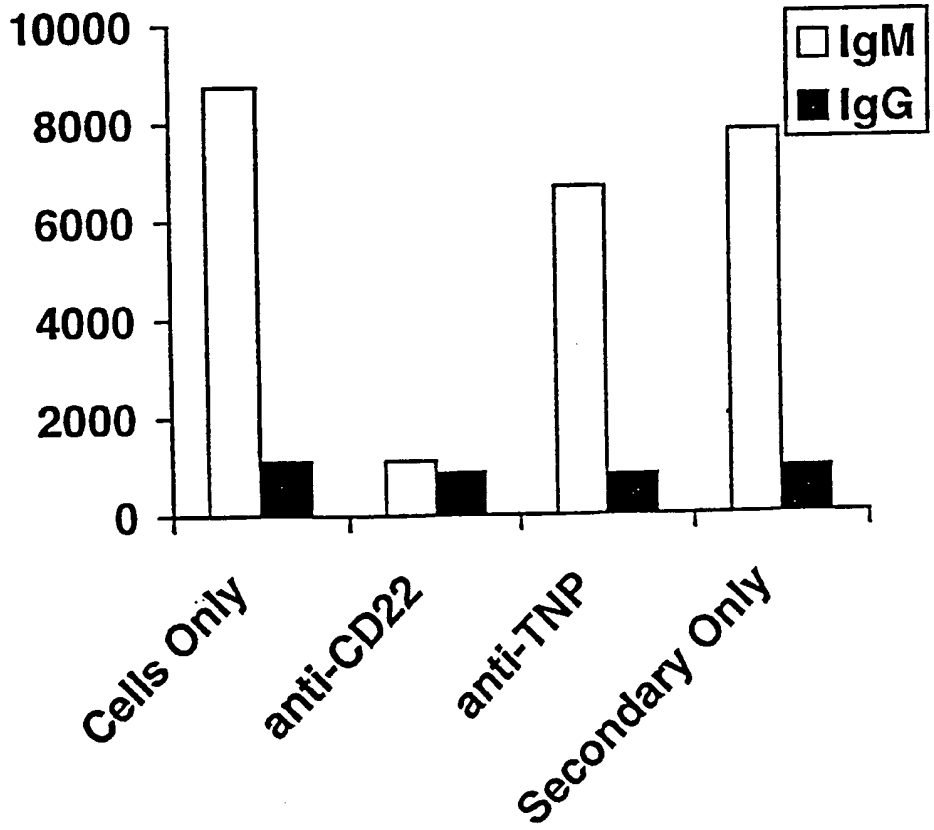
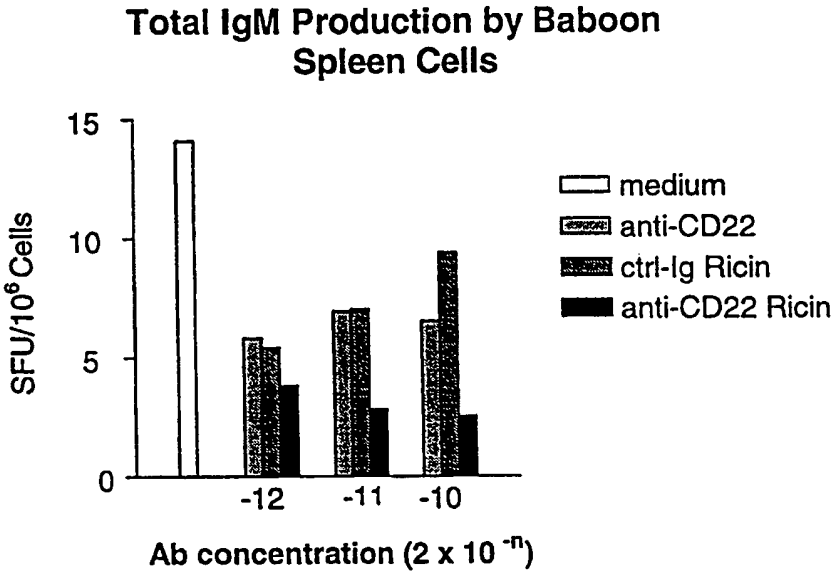


Figure 16


Total IgM Production by Baboon Spleen Cells
15
10
5
0
SFU/10^6 Cells
-12
-11
-10
Ab concentration (2 x 10^-n)
medium
anti-CD22
ctrl-Ig Ricin
anti-CD22 Ricin

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